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Dynamics of the expression of intermediate filaments vimentin and desmin during myofibroblast differentiation after corneal injury

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Abstract

Previous studies have suggested that abnormal corneal wound healing in patients after photorefractive keratectomy (PRK) is associated with the appearance of myofibroblasts in the stroma between two and four weeks after surgery. The purpose of this study was to examine potential myofibroblast progenitor cells that might express other filament markers prior to completion of the differentiation pathway that yields α -smooth muscle actin (SMA)-expressing myofibroblasts associated with haze localized beneath the epithelial basement membrane after PRK. Twenty-four female rabbits that had -9 diopter PRK were sacrificed at 1 week, 2 weeks, 3 weeks or 4 weeks after surgery. Corneal rims were collected, frozen at -80 °C, and analyzed by immunocytochemistry using anti-vimentin, anti-desmin, and anti-SMA antibodies. Double immunostaining was performed for the co-localization of SMA with vimentin or desmin with SMA. An increase in vimentin expression in stromal cells is noted as early as 1 week after PRK in the rabbit cornea. As the healing response continues at two or three weeks after surgery, many stromal cells expressing vimentin also begin to express desmin and SMA. By 4 weeks after the surgery most, if not all, myofibroblasts express vimentin, desmin and SMA. Generalized least squares regression analysis showed that there was strong evidence that each of the marker groups differed in expression over time compared to the other two ($p < 0.01$). Intermediate filaments - vimentin and desmin co-exist in myofibroblasts along with SMA and may play an important role in corneal remodeling after photorefractive keratectomy. The earliest precursors of myofibroblasts destined to express SMA and desmin are detectable by staining for vimentin at 1 week after surgery.

Keywords

photorefractive keratectomy (PRK); myofibroblasts; α -SMA; vimentin and desmin; wound healing

1. Introduction

Photorefractive keratectomy (PRK) is a commonly used refractive eye surgery procedure that uses the excimer laser to correct nearsightedness, farsightedness, and astigmatism in humans. The PRK procedure, or variants such as laser subepithelial keratectomy (LASEK) and Epi-

LASIK, involves damage to or removal of the central corneal epithelium and basement membrane prior to photoablation of Bowman's layer and the anterior stroma (Ambrosio and Wilson, 2003). These techniques are infrequently associated with severe complications such as haze and regression of refractive correction related to abnormalities of corneal wound healing response (Kapadia and Wilson, 2000).

The corneal wound healing process is a complex cascade of events mediated by autocrine and paracrine interactions of cytokines, growth factors, and chemokines produced by epithelial, stromal, and inflammatory cells that contribute to remodeling and reestablishing normal corneal structure and function (Wilson et al., 1999, 2001). One of the early responses to epithelial injury, such as occurs in PRK, LASEK, or Epi-LASIK, is anterior stromal keratocyte apoptosis, followed by the influx of bone marrow-derived cells and the proliferation of residual keratocytes (Zieske et al., 2000; Mohan et al., 2003; Hong et al., 2001; Wilson et al., 2004). As the stromal wound healing response progresses, keratocytes and other cell types, including corneal fibroblasts and, possibly, haze-associated myofibroblasts, repopulate the depleted stroma (Mohan et al., 2003).

When they appear, myofibroblasts contribute to stromal opacity or "haze" associated with corneal wound healing. These cells are traditionally identified through their expression of α -smooth muscle actin (SMA) (Jester et al., 1999a,b; Folger et al., 2001; Netto et al., 2006). Current dogma asserts that myofibroblasts differentiate from keratocytes under the influence of TGF β derived from corneal epithelium (Jester et al., 1999a,b; Folger et al., 2001; Netto et al., 2006), although bone marrow-derived cells have yet to be excluded as alternative progenitors (Netto et al., 2006), as they are in other tissues (Direkze et al., 2003; Hashimoto et al., 2004). Recent studies have demonstrated that structural and functional defects in the regenerated corneal epithelial basement membrane play a critical role in the development of myofibroblasts and haze after PRK, presumably by allowing increased levels of TGF β to penetrate into the anterior stroma and bind to precursor cells (Netto et al., 2006).

One of the enduring mysteries of haze generation in the cornea is the time course of appearance of the haze and associated myofibroblasts. Thus, in humans, severe haze is typically detectable by slit lamp biomicroscopy at around a month after surgery and reaches a peak at approximately three to four months after surgery (Raviv et al., 2000). The slit lamp appearance of haze in rabbits after PRK follows a similar course, although some myofibroblasts that give rise to the haze can be detected by immunocytochemical staining for SMA in the anterior stroma as early as two weeks after the surgical injury (Netto et al., 2006). In other tissues, myofibroblasts have been found to have variable cell phenotypes based on immunohistochemical staining of filaments and a classification system has been proposed (Schmitt-Graff et al., 1994; Kohnen et al., 1996). Thus, myofibroblasts that express only vimentin are termed V-type myofibroblasts, those that express vimentin and desmin are called VD-type, those that express vimentin, SMA, and desmin are called VAD-type, those that express vimentin and SMA are called VA-type, and those that express vimentin and myosin are called VM-type. Nothing is known about these other myofibroblast types in the cornea or whether non-SMA myofibroblasts, for example VD-type, are present beneath the epithelial basement membrane early after corneal injury and give rise to SMA-expressing myofibroblasts that appear only weeks or months later. The purpose of this study was to determine (1) whether vimentin- and/or desmin-expressing stromal cells are present in the rabbit cornea in eyes that are destined to develop haze after PRK and (2) whether cells that express vimentin and/or desmin, but not SMA, are detectable early in the haze-associated corneal wound healing response.

2. Materials and methods

2.1. Animals and surgery

The Animal Control Committee at the Cleveland Clinic approved all of the animal studies included in this work. All animals were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was achieved by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg). In addition, topical proparacaine hydrochloride 1% (Alcon, Ft. Worth, TX, USA) was applied to each eye just before surgery. Euthanasia was performed with an intravenous injection of 100 mg/kg pentobarbital while the animal was under general anesthesia.

Twenty-four 12- to 15-week-old female New Zealand white rabbits weighing 2.5-3.0 kg each were included in this study. One eye of each rabbit was selected at random to receive -9 diopter (D) photorefractive keratectomy (PRK) with a 6.0 mm ablation zone using an Apex Summit Laser (Alcon, Ft. Worth, TX). Our previous studies have shown that 100% of rabbit corneas receiving this level of PRK treatment with this laser develop severe late stromal haze (Mohan et al., 2003). Four groups of 6 animals each were included in this study and animals were divided based on the number of days prior to sacrifice after PRK treatments. Thus, six animals were included in the 1-week, 2 weeks, 3 weeks and 4 weeks groups, respectively.

2.2. Cornea collection and tissue preparation

Rabbits were euthanized and the corneoscleral rims of ablated and unablated control eyes were removed with 0.12 mm forceps and sharp Westcott scissors. For histological analyses, the corneas were embedded in liquid OCT compound (Sakura FineTek, Torrance, CA, USA) within a 24 mm × 24 mm × 5 mm mould (Fisher, Pittsburgh, PA, USA). Cornea specimens were centered within the mould so that the block could be bisected and transverse sections cut from the center of the cornea. The mould and tissue were rapidly frozen and stored at -80 °C until sectioning was performed.

Central corneal sections (7 µm thick) were cut with a cryostat (HM 505M, Micron GmbH, Walldorf, Germany). Sections were placed on 25 mm × 75 mm × 1 mm microscope slides (Superfrost Plus, Fisher) and maintained frozen at -80 °C until staining was performed.

2.3. Immunocytochemistry in corneal tissues

α-Smooth muscle actin (α-SMA) was detected using a monoclonal mouse anti-human smooth muscle actin clone 1A4 (Dako, Carpinteria, CA) at concentration of 1:50 for 90 min in 1% BSA. The initial secondary antibody, Alexa Fluor 488 (Invitrogen, Carlsbad, California) goat anti-mouse IgG (H + L) (green) at a concentration of 1:100 for 60 min in 1% BSA. Sections were then incubated for 60 min with mouse normal serum (Jackson ImmunoResearch, West Grove, PN) diluted 1:5 with phosphate buffered saline (PBS). After washing with PBS, a second incubation was performed with excess unconjugated goat Fab antibody against mouse for 60 min. Double immunostaining was performed to co-localize vimentin with SMA and desmin with SMA. The monoclonal mouse anti-vimentin clone Vim 3B4 (Dako) or anti-Desmin clone D33 (Dako) was used at room temperature for 60 min. The working concentrations for anti-vimentin and anti-desmin were 1:200 and 1:20 in 1% BSA, respectively and were selected after titration experiments to limit non-specific staining (for example, of unwounded corneal epithelium by vimentin antibody). The secondary antibody, Alexa Fluor 568 goat anti-mouse IgG (H + L) (red) was applied at a concentration of 1:100 for 60 min. Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA) to allow visualization of all nuclei in the tissue sections. Negative controls were included with secondary antibody alone. The sections were viewed and photographed

with a Leica DM5000 microscope equipped with Q-Imaging Retiga 4000RV (Surrey, BC, Canada) camera and ImagePro software.

2.4. Quantification of cells

The SMA-, vimentin- and desmin-positive cells were counted from six different corneas in the six randomly selected, non-over-lapping, full-thickness central corneal columns under 400 $\times$ microscopic field, as previously described (Mohan et al., 2003).

2.5. Statistical analysis

Statistical analyses were performed by a biostatistician (SDS). Quantitative data for each marker were presented with box and whisker plots. The line in the center of each box is the median and the top and bottom of the boxes are the 25th and 75th percentiles. The distance between the 25th and 75th percentiles (the length of the box) is the interquartile range. The top and bottom of the “whiskers” are at the “upper and lower adjacent values” which represent the points furthest from the median that fall within 1.5 times the interquartile range from the median. The points beyond the end of the whiskers are outliers (by definition, points that fall >1.5 times the interquartile range from the median).

The relationship between the mean cells stained/400 $\times$ field for each marker evaluated and the time following treatment was analyzed using generalized least squares linear regression models. This method accounts for correlations within the data, allowing the use of all observations from each animal within each regression model. Time (in weeks) following treatment was used as a predictor of the mean cell counts for vimentin, α -smooth muscle actin and desmin. The slope associated with time since treatment was considered to be statistically significant if its associated *p*-value was <0.05.

3. Results

Importantly, individual staining was performed on all corneal specimens with antibodies to SMA, vimentin or desmin prior to performing double-labeling experiments. Results for individual antigens were identical to those found in double-labeling experiments, but single staining images are not shown because they are redundant to the staining shown in the Figs. 1 and 2.

3.1. Identification, localization and distribution of vimentin and SMA in unwounded and wounded rabbit corneas

Vimentin expression in the rabbit cornea is illustrated in Fig. 1. Starting from the left to right, the columns show fluorescence images stained with DAPI or with immunocytochemistry for vimentin or SMA. The last column on the right shows overlays of all three stains together. Representative images are shown for each stain at each time point. Analysis of normal unwounded rabbit corneas frozen section with anti-vimentin antibody (Fig. 1A) demonstrated low levels of vimentin expression in the anterior stroma whereas anti-SMA antibody did not react with any stromal cells in the unwounded cornea. At one-week after PRK wounding (Fig. 1B), the number of vimentin+ cells has increased in the area directly beneath the epithelium. Further increase in the numbers of vimentin+ cells and the intensity of staining of individual stromal cells is noted at 2 weeks (Fig. 1C) and 3 weeks (Fig. 1D) after PRK. In contrast, the earliest detection of SMA+ cells in the stroma was at 2 weeks after PRK. By 3 weeks (Fig. 1D), there was an increase in the staining pattern of the stromal fibroblasts with vimentin in the wounded areas, accompanied by a gradual increase in the expression of SMA in stromal cells. Careful examination of double staining for vimentin and SMA at 3 weeks after PRK (Fig. 1D), and especially at 4 weeks after PRK (Fig. 1E), reveals that some cells in the anterior stroma simultaneously express both vimentin and SMA. There are numerous stromal cells in

the anterior stroma that express vimentin, but not SMA, at 3 weeks after PRK (Fig. 1D). We did not identify cells in the corneal stroma at any time point that were SMA+, but vimentin-. At four weeks after PRK (Fig. 1E), vimentin localization in stromal cells is almost identical to SMA localization, with almost all cells that are vimentin+ also being SMA+, although the level of SMA expression in individual cells increased at 4 weeks compared to 3 weeks after PRK.

3.2. Identification, localization and distribution of desmin and SMA in unwounded and wounded rabbit corneas

Desmin and SMA expression in wounded and unwounded rabbit corneas are shown in Fig. 3. We could not detect the expression of desmin in the fresh frozen sections of unwounded control rabbit corneas or first two weeks after wounding (Fig. 2A-C). A significant number of desmin-positive cells were identified in the anterior stroma of corneas by the third week after PRK (Fig. 2D). By four weeks after PRK, there were numerous desmin+ cells in the anterior stroma (Fig. 2E). The level of desmin expression in stromal cells lagged behind SMA and all cells that were desmin+ also appeared to be SMA+ throughout the 4 weeks of healing after PRK that were monitored in this study (Fig. 2). By four weeks after PRK, stromal cells beneath the epithelial basement membrane, where myofibroblasts have been localized in this model, express both desmin and SMA at high levels (Fig. 2E), with apparently 100% concordance of expression.

3.3. Quantitative evaluation of vimentin, α -smooth muscle actin and desmin expression over time after 9 diopter PRK for myopia

Box and whisker plots for vimentin, α -smooth muscle actin and desmin are shown in Fig. 3A-C, respectively. Note that each marker increases over time to four weeks. Vimentin was detected in stromal cells in the unwounded cornea (Figs. 1 and 3A). Neither SMA nor desmin was detected in stromal cells in the unwounded cornea (Figs. 1-3B and C). Generalized least squares regression analysis showed that there was strong evidence that each of the marker groups differed in expression over time compared to the other two ($p < 0.01$).

4. Discussion

The late occurrence of opacity and myofibroblasts associated with late haze after photorefractive keratectomy (PRK) or other injuries to the corneal stroma has never been adequately explained. We hypothesized that myofibroblast precursors were present in the stroma immediately after injury, but must undergo a differentiation program, driven by cytokines such as TGF β and PDGF that penetrate into the stroma in the context of epithelial basement membrane structural and functional defects, that lead to the expression of α -smooth muscle actin (SMA) - the marker traditionally used to detect myofibroblasts. In the present study in rabbits, we demonstrate that an increase in stromal vimentin-expressing cells (likely V-type myofibroblasts) precedes the appearance of myofibroblasts that also express SMA (VA-type myofibroblasts). Some of these V-type cells are detectable beneath the epithelial basement membrane in the unwounded cornea (Fig. 1A vimentin) and could represent the earliest precursors to myofibroblasts that are activated by cytokines after injury. Slightly later in the wound healing process, at approximately 2 weeks, cells that express desmin also appear in the stroma, but all of these cells appear to already express SMA (Fig. 3C desmin and SMA). From the data in Figs. 1 and 3, it appears that most of these cells also express vimentin and, therefore, are VAD-type myofibroblasts, but we were unable to conclusively demonstrate this with triple stain immunocytochemistry due to technical issues with the antibodies. Thus, it would appear that myofibroblast precursors undergo an ordered differentiation from V-type cells, to VA-type cells to VAD-type cells during the healing response to corneal injuries associated with haze.

Previous studies of vimentin expression in alkali burned and incised corneas found more uniform vimentin staining throughout the stroma, including in unwounded corneas (Ishizaki et al., 1993). In preliminary experiments with the primary antibody for vimentin used in this study, some staining of posterior keratocytes was occasionally noted with higher primary antibody concentrations. However, the differential staining pattern showing greater vimentin expression in the most anterior keratocytes was consistently noted. Thus, while more posterior keratocytes may express smaller levels of vimentin in the unwounded cornea, the results of this study suggest that there is a phenotypic difference in vimentin expression between anterior stromal and posterior stromal keratocytes in the unwounded rabbit cornea.

The intermediate filaments vimentin and desmin, along with actin microfilaments, microtubules, and their associated protein constitute a cytoskeleton system in the human cornea. These components interact with each other to form a meshwork essential for the normal growth, differentiation, integrity, and function of corneal cells (Kivela and Uusitalo, 1998). Different classes of intermediate filaments are known to co-exist in the same cell during various developmental stages and the profile may change during proliferation, stress, and malignant transformation (Cooper et al., 1985; Klymkowsky et al., 1989; Paulin-Levasseur, 1992).

Vimentin is known as the major class of the intermediate filaments of mesenchymal cells such as fibroblasts, endothelial cells, melanocytes, and adipocytes (Lazarides, 1980). On the other hand, desmin is present in striated muscle cells, myocardium, and most smooth muscle cells (Lazarides, 1980). In the muscle cells, desmin is known to strengthen and maintain cell integrity and desmin expression levels depend on the amount of stress or stretch applied to these cells (Watson et al., 1996; Li et al., 1996). In astrocytes, vimentin is known to directly co-polymerize with desmin and glial fibrillary acidic protein to stabilize the filaments (Galou et al., 1996). Although there is basal expression of vimentin in keratocytes in the cornea (Ishizaki et al., 1993; Wollensak and Witschel, 1996; Kivela and Uusitalo, 1998; Mimura et al., 2008), constitutive expression of desmin is not observed in keratocytes in the control unwounded cornea (Ishizaki et al., 1993; Kivela and Uusitalo, 1998). Our results *in situ* in rabbit unwounded control corneas confirm these observations. Desmin is a minor component of the intermediate filament network and it is possible it is present in the unwounded keratocytes at low, undetectable levels using the sensitive immunocytochemical methods used in this study. After the PRK surgery, however, a strong fluorescent signal was observed. This was likely related to stress and reorganization of myofibroblasts during the wound healing response. Desmin is known to form homopolymers or heteropolymers with vimentin (Klymkowsky et al., 1989) and it likely plays an important role in strengthening and maintaining the integrity of the myofibroblasts and, therefore, the corneal stroma itself, as was reported earlier in muscle tissues (Watson et al., 1996; Li et al., 1996). Thus, after a corneal injury that provides sufficient stimulus and results in basement membrane dysfunction, there is transdifferentiation and rearrangement in the cytoskeleton architecture of the corneal stromal cells which results in increased expression of vimentin, and subsequently SMA and desmin, in cells of the stroma in proximity to the injury. This contributes to added stromal strength and, in the case of incisional injuries, contraction of the wound.

Keratocytes of the corneal stroma are broad, flattened fibroblast-like cells that lie parallel to the collagen lamellae to extend cellular processes interconnecting each other at occasional desmosome-like junctions (Wilson et al., 2001; West-Mays and Dwivedi, 2006). Corneal wound healing in context of PRK surgery results in the removal of defined amount of stromal tissue to sculpt the cornea into a defined and predictable surface contour (Wilson, 2002). While normal stromal keratocytes appear quiescent, injury to cornea induces a series of changes in the corneal architecture, including the classical apoptosis of keratocytes underlying the site of epithelial injury, within few hours after injury (Wilson et al., 1999, 2007; Wilson, 2002; West-Mays and Dwivedi, 2006). One of the important events in the corneal wound healing response

is the subsequent cellular repopulation of the affected stroma. Stromal cells are replenished through migration of residual keratocytes and bone marrow-derived cells and proliferation of at least some of these cells. Some stromal cells are activated under the influence of cytokines such as TGF β and PDGF released from the epithelium. These events lead to the development of fibroblastic cells having ultrastructural, biochemical, and physiological properties of myofibroblasts. Myofibroblasts appear to control stromal remodeling through deposition and organization of extracellular matrix in corneal wounds and are responsible for corneal wound contraction (Masur et al., 1996; Fini, 1999; Jester et al., 1999b; Kuo, 2004; Wilson et al., 2007). Expression of SMA, a smooth muscle-specific protein, has been used as a molecular marker for myofibroblast transformation. In the present study, SMA was largely expressed beneath the epithelial basement membrane at the site of injury and showed maximal stain at the latest 4-week time point monitored in this study. Expression of SMA could have been even higher at later time points, for example 2 and 3 months, since haze continues to intensify in at least some animals up to this time point, prior to gradual resolution mediated by late apoptosis of the myofibroblasts and reabsorption and reorganization of matrix materials produced by these cells (Wilson et al., 2007).

A major question that remains unanswered from this investigation and earlier studies (Jester et al., 1999a,b; Wilson, 2002; Wilson et al., 2007) is whether all corneal myofibroblast cells are derived from keratocytes or bone marrow-derived cells can also serve as precursor cells for corneal myofibroblasts, as they are in other organs (Bhawan and Majno, 1989; Direkze et al., 2003; Hashimoto et al., 2004). We have attempted to conclusively exclude this possibility using mouse fluorescent green protein technology, but the results have been equivocal. Now that a method to generate haze in mice has been developed (Mohan et al., 2008) we are once again exploring this question.

In summary, stromal cells that develop in the wounded area of the anterior corneal stroma during the first two weeks after PRK express only vimentin, and hence are V-type myofibroblasts. At least some of these cells further differentiate to express SMA and/or desmin and, therefore are VA- and/or VAD-type myofibroblasts associated with stromal haze.

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Fig. 1.

Immunocytochemistry for vimentin and α -smooth muscle actin in the unwounded rabbit corneal stroma (A) and at 1 week (B), 2 weeks (C), 3 weeks (D), and 4 weeks (E) after -9 diopter photorefractive keratectomy. Six corneas were studied at each time point and the panels show representative results. Cells nuclei are stained blue with DAPI, SMA-positive cells are stained green and vimentin-positive cells are stained red. Representative positive-stained cells are indicated with arrows in some panels. Note that in the unwounded cornea there are vimentin+ cells in the stroma beneath the epithelial basement membrane. These vimentin+ cells increase in number and have increased levels of vimentin at 1, 2, 3, and 4 weeks after PRK (arrows B, C, D, and E). SMA is not detectable until 2 weeks after PRK (arrows). In the double-stained column of C, D, and E, it can be seen that many (arrows E DAPI/vimentin/SMA), but not all, cells in the anterior stroma are positive for both vimentin and SMA. Magnification 400 $\times$. The bar in DAPI staining E shows 50 μ m. With cryopreservation of rabbit corneas it is common during sectioning to have artifactual separation of the epithelium from the underlying stroma. White vertical bars in the DAPI image for each week delineate this separation, if any, that is also present in the other images for that week. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Immunocytochemistry for α -smooth muscle actin and desmin in the unwounded rabbit corneal stroma (A) and at 1 week (B), 2 weeks (C), 3 weeks (D), and 4 weeks (E) after -9 diopter photorefractive keratectomy. Six corneas were studied at each time point and the panels show representative results. Cells nuclei are stained blue with DAPI, SMA-positive cells are stained green and desmin-positive cells are stained red. Representative positive-stained cells are indicated with arrows in some panels. Note that no SMA or desmin were detected in the unwounded corneas or at 1 week after PRK (A and B). At two weeks (C) and 3 weeks (D) after PRK, many SMA-positive cells could be detected beneath the epithelial basement membrane. Some of these cells, if not all, were also desmin+, although in some desmin was expressed at low levels, if at all. By four weeks after PRK (E) there appeared to be 100% concordance between SMA and desmin expression in the anterior stromal myofibroblast cells. Magnification 400 $\times$. The bar in DAPI staining E shows 50 μ m. With cryopreservation of rabbit corneas it is common during sectioning to have artifactual separation of the epithelium from the underlying stroma. White vertical bars in the DAPI image for each week delineate this separation, if any, that is also present in the other images for that week.

Fig. 3.

Box and whisker plots for vimentin (A), α -smooth muscle actin (B) and desmin (C) expression over time after 9 diopter PRK for myopia in rabbits. The line in the center of each box is the median and the top and bottom of the boxes are the 25th and 75th percentiles. The distance between the 25th and 75th percentiles (the length of the box) is the interquartile range. The top and bottom of the “whiskers” are at the “upper and lower adjacent values” which represent the points furthest from the median that fall within 1.5 times the interquartile range from the median. Note that vimentin was the only marker of the three that was detected in stromal cells (presumably keratocytes) in the unwounded cornea (data from the unwounded corneas are not shown in C for desmin, but none was detected, identical to week 1).